Journal of Virological Methods 193 (2013) 238–243

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Expression of a truncated hepatitis E virus capsid protein in the protozoan organism *Leishmania tarentolae* and its application in a serological assay

C. Baechlein[a,*], D. Meemken[b], G. Pezzoni[c], C. Engemann[d], B. Grummer[a]

[a] Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Germany
[b] Institute for Food Quality and Food Safety, University of Veterinary Medicine Hannover, Germany
[c] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy
[d] QIAGEN Leipzig GmbH, Leipzig, Germany

Article history:
Received 4 January 2013
Received in revised form 14 May 2013
Accepted 28 May 2013
Available online xxx

Keywords:
Zoonosis
Pig
Antigen expression
Leishmania tarentolae
Serology

A B S T R A C T

Zoonotic infections with hepatitis E virus (HEV) genotype 3 are presumably transmitted via contaminated pig meat products, which raises the necessity for enhanced serological surveillance of pig herds. The aim of the study was to set up a novel protein expression system to overcome the well-known problems in (HEV-) protein expression using the standard *Escherichia coli* tools such as inclusion body formation and loss of protein conformation. A recombinant strain of the protozoan organism *Leishmania tarentolae* (*L. tarentolae*) was therefore established. A fragment of HEV ORF2 coding for a truncated capsid protein of a porcine HEV strain was cloned and parts of the plasmid DNA were introduced into the Leishmania genome, resulting in stably transformed cells. Via a C-terminal His-tag the recombinant HEVΔORF2 protein could be purified and concentrated directly from the medium, resulting in a total protein amount of approximately 1.4 mg/l Leishmania culture. The recombinant protein was coated on ELISA plates and was proven to be highly reactive and well-suited to be applied in a serological assay. By investigating 144 porcine sera, the in-house assay detected specific antibodies in 43.1% of the samples and demonstrated a higher sensitivity than a commercially available antibody test. Taken together, it was shown that *L. tarentolae* exhibits a remarkable alternative expression strategy for viral antigens with considerable advantages of a eukaryotic protein expression host.

1. Introduction

Hepatitis E virus (HEV), a non-enveloped RNA virus is responsible for large water-borne outbreaks of acute hepatitis in different parts of the world, mainly in developing regions where HEV genotypes 1 and 2 are predominant. In industrial countries, hepatitis E is believed to be caused mainly by a zoonotic infection with HEV genotypes 3 and 4 (Aggarwal, 2011). In the case of Germany, there is a growing body of evidence for high HEV seroprevalences in the adult population (Faber et al., 2012). In addition to acute infections, HEV genotype 3 is known to cause persistent disease in immunocompromised patients such as organ transplant recipients (Kamar et al., 2008; Legrand-Abravanel et al., 2010; Pischke et al., 2012). Certainly, wild boars as well as domestic pigs serve as reservoir hosts in Europe (Pavio et al., 2010) and several reports support the possibility of HEV transmission via pork food products (Colson et al., 2010; Wenzel et al., 2011).

The prevalence of anti-HEV antibodies in domestic pigs was investigated largely in the past few years (Baechlein et al., 2010; Breum et al., 2010; Peralta et al., 2009; Rose et al., 2011; Seminati et al., 2008; Wacheck et al., 2012). In serological assays, the viral capsid protein, which is encoded by the second open reading frame (ORF2) was most often used as antigen since it is known to harbour several neutralizing epitopes (Meng et al., 2001). To date, various expression systems for HEV antigens have been used to set up serological tests including the use of prokaryotic as well as eukaryotic organisms (Dremsek et al., 2012; Jiménez de Oya et al., 2009; Peralta et al., 2009). In addition to this, the aim was to establish an expression system which combines all the benefits of the yet recognized approaches.

The non-human pathogenic species *Leishmania tarentolae* (*L. tarentolae*) is a protozoan parasitic organism which was found to infect naturally the lizard *Tarentola annularis* (Elwasila, 1988). In 2002, Breitling et al. generated a protein expression system based on

* Corresponding author at: Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany. Tel.: +49 511 953 8845.
E-mail address: christine.baechlein@tiho-hannover.de (C. Baechlein).

0166-0934/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2013.05.018

the stable transformation of *L. tarentolae* cells, which ensures the constant expression of foreign proteins after homologous recombination of the respective DNA with the Leishmania small subunit RNA gene. In this case, the signal peptide for secreted acid phosphatase of *L. mexicana* ensures transportation of the recombinant protein to the medium (Breitling et al., 2002). Since then, a broad range of proteins have been produced via this expression system (Basile and Peticca, 2009). Apart from the ability to form native disulfide bonds, the strong resemblance of the N-linked glycosylation pattern of *L. tarentolae* in particular to the glycosylation form of mammalian cells is of considerable interest when conformation becomes crucial for protein functionality (Phan et al., 2009; Soleimani et al., 2007). In addition, protein structure is extremely important when investigating antigen-antibody binding reactions. In particular for HEV it was shown that neutralizing sites are characterized by discontinuous epitopes (Zhou et al., 2005; Zhang et al., 2012). Therefore, in the present study, the *L. tarentolae* expression system was used to produce a novel HEV antigen and to explore if it could be advantageous to established recombinant HEV proteins.

2. Materials and methods

2.1. Cloning of the HEV fragment

Total RNA was isolated from an HEV genotype 3 positive liver sample derived from a domestic pig. Complementary DNA (cDNA) was synthesized with random primers and SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). Next, primers HEVΔORF2_XbaI (5′-CTGCGCCTCTAGACGCCATATCACCTGCTCCCGAT-3′) and HEVΔORF2_KpnI (5′-TCTTCAAGGGTACCAAGAGCCGAATGTGGG GCTAAAA-3′) were used to amplify a 1516 bp long fragment of the HEV ORF2. The restriction sites required for in-frame-cloning into the vector pLexsyHyg2 (Jena Bioscience, Jena, Germany) are underlined. After purification of the PCR product, a treatment with *Xba*I and *Kpn*I ensured a correct ligation with the help of the *T4* ligase (all enzymes from Thermo Fisher Scientific, Waltham, MA, USA). After ligation overnight at 15 °C the construct was introduced into *Escherichia coli* SURE Electroporation-Competent Cells (Agilent Technologies, Santa Clara, CA, USA). Ampicillin resistant clones were confirmed by sequencing to carry the plasmid with the desired fragment.

2.2. Leishmania culture and transformation

L. tarentolae strain P10 (Jena Bioscience, Jena, Germany) was cultured in brain heart infusion (BHI) medium (37 g/l) with penicillin (50 U/ml), streptomycin (50 μg/ml) and hemin (5 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) at 27 °C according to the manufacturer's instructions. To select for recombinant cells, hygromycin was added to a final concentration of 50 μg/ml (PAA Laboratories, Pasching, Austria). To maintain a culture, cells were passaged twice a week with dilutions of 1:40.

To transfer the DNA into the Leishmania cells, 20 μg of the plasmid pLexsyHyg2_HEVΔORF2 were digested with *Swa*I. 3 ml of a two-day-old culture were centrifuged at 330 × *g* for 7 min and resuspended in 1 ml of *cytomix* electroporation buffer (Van den Hoff et al., 1992). Of these, 350 μl were mixed with 50 μl digested plasmid DNA and kept on ice for 10 min. Cells were then transferred to a 4.0 mm cuvette (Bio-Rad, Hercules, CA, USA) and electroporated by two pulses (0.3 ms) at 1500 V. After 10 min incubation on ice, electroporated cells were transferred to 8 ml of BHI-medium.

2.3. Clonal selection of recombinant cells and verification of protein expression

Two days after electroporation, 2 ml of the culture were pelleted by centrifugation and resuspended in 80 μl medium. They were spread on agar plates containing BHI, antibiotics, hemin, the selection marker hygromycin, and 10% foetal bovine serum as well as 1 M HEPES. Plates were sealed and incubated upside-down at 27 °C. Approximately one week later, individual colonies were picked and transferred into 100 μl BHI medium containing hygromycin. Subsequently, proliferating cells were grown and scaled up before storage as glycerol stocks at −80 °C.

Two pairs of primers were used to prove the genomic integration of the expression cassette. After DNA preparation, the primers A3804 (5′-CCGATGGCTGTGTAGAAGTACTCG-3′) and F3002 (5′-CTGCAGGTTCACCTA CAGCTAC-3′) (Jena Bioscience, Jena, Germany) were applied in the first PCR reaction to verify the integration of the fragment including the marker gene into the chromosomal 18S rRNA locus (*ssu*). Thereby, A3804 binds to the hygromycin phosphotransferase gene whereas F3002 attaches to the *ssu* outside the site of recombination. To prove the specificity of the inserted DNA, a second PCR reaction was performed with the primers F3001 (5′-GATCTGGTTGATTCTGCCAGTAG-3′) binding to the Leishmania *ssu* and a particular HEV ORF2 primer HEV5826 (5′-TAGAGTTCATATCAACAGACGTGGG-3′).

To investigate the protein expression, total protein was precipitated from 9 ml of the supernatant by 1.8 ml trichloroacetic acid followed by centrifugation at 15,000 × *g* and 4 °C. The pellet was washed with 80% acetone and resuspended in 2× SDS sample buffer. After SDS gel electrophoresis and Western blotting the HEVΔORF2 protein was detected with mouse anti-His_6 (Roche Applied Science, Penzberg, Germany) or with mouse anti-HEV (United States Biological, Salem, MA, USA). Polyclonal rabbit anti-mouse immunoglobulin/HRP (Dako, Glostrup, Denmark) was applied as secondary antibody in both cases.

2.4. Expression and purification of the HEVΔORF2 protein

A frozen glycerol stock of a recombinant clone was thawed on ice and transferred into BHI medium complemented with 50 μg hygromycin/ml medium. When the culture had reached an optical density of about 1.5, cells were separated from the medium by centrifugation for 20 min at 4000 × *g*. Protein purification was carried out under native conditions using a liquid chromatography system and a column pre-packed with Ni-NTA agarose (Macherey-Nagel, Düren, Germany). Subsequently, the protein was eluted from the column with a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazol and was stored at 4 °C.

2.5. ELISA development and comparison to a commercially available test

To investigate the reactivity of the novel antigen, different amounts of the ΔORF2 antigen ranging from 200 ng/well to 6.25 ng/well were tested. For this, 96-well plates (Medisorp, Thermo Fisher Scientific, Waltham, MA, USA) were coated at room temperature or 37 °C in a 0.1 M carbonate/bicarbonate buffer. Further on, seven anti-HEV-IgG-negative as well as seven positive serum samples were tested in different concentrations. The antibody status of these samples was previously determined by ELISA based on a recombinant ORF2 protein expressed via a baculovirus system. For the detection of specific serum IgG antibodies, rabbit anti-pig IgG peroxidise conjugate (Sigma–Aldrich, St. Louis, MO, USA) was applied, followed by an incubation step with the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Promega, Fitchburg, WI, USA).